



Effect of epiphytic lactic acid bacteria fermented juice inclusion on quality of total mixed ration silage of agricultural and food by-products

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ABSTRACT

Present study determined the effect of fermented juice of epiphytic lactic acid bacteria (FJLB) as silage additive on the fermentation quality and nutritive value of total mixed ration (TMR) silage prepared from agricultural and food industrial by-products, to compare the resultant silage quality with that of silage produced with commercial additive. FJLB was prepared from Italian ryegrass. The applied treatments were: (i) CON (Control-no additive); (ii) FJLB (as silage additive); (iii) COM (commercial silage additive); and (iv) MIX (combination of FJLB and commercial additive). The fermentation quality was measured at 2, 7, 14, 30 and 60 days of fermentation. The nutritive value was measured at 60 day of fermentation. The addition of the FJLB to TMR produced a better quality silage in terms of a lower pH, higher lactic acid content, lower dry matter (DM) loss and higher Fleig point than occurred with COM and CON treatments. The addition of the FJLB combined with the commercial silage additive decreased the aNDFom and ADFom content of the TMR silage. In conclusion, inclusion of the FJLB to the TMR silage prepared from agricultural and industrial by-products improved the fermentation quality.

Key words: Agricultural by-product, Epiphytic lactic acid bacteria, Fermentation quality, TMR silage

Production of agricultural by-product is increasing with increase in crop production in the entire world (FAO 2015). In few tropical countries, animal production depends on agricultural by-products (Van Soest 2006) owing to the inadequacy of forage grasses. However, agricultural by-products are known for low nutrient content and poor digestibility, leading to low animal production. Thus, efforts to improve the utilization of agricultural by-products as feed in ruminants are being made for decades (Yanti and Yayota 2017). The combination of agricultural by-products and other nonstructural carbohydrates in the form of total mixed ration (TMR) seems to be a promising method to improve the utilization of these feed in ruminants. Ensilaging of TMR is usually carried out to avoid the loss of quality during transportation and storage for extended periods. Several studies suggested that making TMR silage that contains agricultural by-products may improve the digestibility of silage in animals (Cao *et al.* 2016, FAO 2012), thus, TMR silage is a possible way to use agricultural by-products for ruminant feed. Furthermore, using high-quality silage is also a key factor in diminishing the cost of production and maintaining animal health (Wanapat *et al.* 2013).

The use of additives to prepare silage has been employed for decades to achieve a good fermentation quality of the

silage because high-quality silage fed to animals can sustain animal health and reduce the cost of production (Kang *et al.* 2018). One possible silage additive is the fermented juice of epiphytic lactic acid bacteria (FJLB) or previously fermented juice (Ohshima *et al.* 1997, Bureenok *et al.* 2005). Use of FJLB seems more likely to be implemented on farms of any scale because FJLB is easy to prepare and has a negligible cost (Nishino and Uchida 1999).

The application of FJLB resulted in good fermentation of napier grass (Tamada *et al.* 1999), Guinea grass and stylo legume (Shao *et al.* 2004, Bureenok *et al.* 2005, 2016). However, the limited studies on application of FJLB to agricultural by-products have been carried out, where rice straw (Jin-ling *et al.* 2013) and whole crop residues (Takahashi *et al.* 2005) sprayed with FJLB increases the crude protein (CP) content and reduces the dry matter (DM) losses. Present study hypothesized that the application of FJLB in ensiling may also improve the quality of the TMR silage prepared from the agricultural and food by-products. Therefore, the aim of the present study was to improve the utilization and quality of agricultural and food by-products using FJLB as a silage additive and to compare the fermentation quality and nutritive value of the TMR silage with that of the silage produced with commercial additive.

MATERIALS AND METHODS

Ingredients: TMR silage was prepared using rice straw, corn cobs, brewer grain waste, tofu waste, steam-flaked

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corn and vitamin-mineral mix. The rice straw chopped into 2–3 cm length pieces before ensiling, brewer grain waste and tofu waste, cut corn cobs and steam-flaked corn were obtained from a local feed companies (Minorakuren; and Oriental Yeast Co., Ltd., Gifu, Japan). Ingredients were mixed and a dry TMR was prepared with vitamin-mineral mix. The vitamin-mineral mix (NAS DL05-HVE; NASU AGRI SERVICE, Inc., Tokyo, Japan) contained 5,000,000 IU/kg of vitamin A, 1,000,000 IU/kg of vitamin D, 24,000 IU/kg of vitamin E, 150 mg/kg of Co, 8,000 mg/kg of Cu, 15,000 mg/kg of Mn, 250 mg/kg of I, 20,000 mg/kg of Zn, 10 mg/kg of Se, and 700 g/kg of Mg. The proportions of ingredients were fixed at 23, 23, 20, 14.5, 18 and 1.5 g/100 g TMR (DM) for rice straw, corn cobs, tofu waste, brewer grain waste, steam-flaked corn and vitamin-mineral mix, respectively. The TMR was designed for sheep and was formulated to obtain 12.4% CP and 65.9% total digestible nutrients (TDN) to meet or exceed the nutrient requirements of sheep (NRC 2007).

Preparing the silage: A 500 g DM of TMR was placed in polyethylene silo bags (420 mm × 500 mm) for different treatments, namely TMR: no additive (CON); FJLB; commercial additive, 'Si-Master AC' (COM); and MIX (FJLB + commercial additive). The FJLB was prepared from Italian ryegrass before harvesting using modified protocols from Burenook *et al.* (2016). The LAB in FJLB increased from 1.1×10^4 CFU/ml to 4.7×10^7 CFU/ml after incubation (enumerated using MRS agar methods). The FJLB was added at a rate of 1% (v:w) to fresh material. The commercial additive was diluted with distilled water (17:1,000 ratio; w:v) and sprayed at a rate of 0.1% (v:w) on the TMR according to the company's instructions. Then, distilled water was added to all the silo bags to obtain a 55% DM content. Five replicates of all the silages were made for each treatment for each fermentation period. The silo bags were kept at ambient temperature (25.3–32.6°C), and samples were taken at 2, 7, 14, 30, and 60 days of ensiling to measure the quality of the fermentation. The chemical analysis of the TMR silages was conducted after 60 days of fermentation.

Silage sampling and fermentation quality measurement: On each sampling day, a representative 50 g of fresh matter from the ensiled sample was macerated with 150 ml of distilled water and stored at 4°C for 12 h (Burenook *et al.* 2016). The extract was filtered using filter paper (ADVANTEC No. 1, Tokyo, Japan) and filtrate was used to determine the fermentation quality. The pH of the silage was determined using a pH meter (MP220; Mettler-Toledo, Tokyo, Japan) immediately after filtration and volatile fatty acids (VFA) were measured by gas chromatography (GC-14A, Shimadzu, Kyoto, Japan; column: ULBON HR-20M, 0.25 mm I.D. × 30 mL 0.25 µm; flow rate: 7.2 ml/min; injection port temperature: 220°C; column temperature: 200°C; and detector temperature: 220°C). The lactic acid content was determined using a commercial kit (D-/L-Lactic Acid Assay Kit; Megazyme, Wicklow, Ireland) and the NH₃-N level was measured using indophenol method

(Weatherburn 1967). The Fleig points were measured using the mathematical equation (Denek and Can 2006):

$$\text{Fleig points} = 220 + (2 \times \text{DM}\% - 15) - 40 \times \text{pH}.$$

The IVDMD was analyzed by the batch culture method according to Tilley and Terry (1963). Rumen fluid was collected from 2 cannulated Suffolk sheep before feeding. Sudan grass hay and concentrate were fed to the sheep at the maintenance level, and equal amounts of the feed were offered in the morning and evening. The rumen fluid was then filtered with 4 layers of cheesecloths and mixed with anaerobic McDougal buffer (1:4, v:v). A 25 ml aliquot of this mixture was mixed with 0.25 g of sample in a 50 ml glass vial with a rubber cap and gas trap. The glass vials were incubated in a shaking waterbath at 39°C for 48 h.

Chemical analyses: A representative sample of silage (120 g) was dried at 60°C to a constant weight and then ground <1 mm for chemical analyses. The DM, ash, acid detergent fiber exclusive ash (ADFom), CP, and ether extract (EE) analyses were performed according to AOAC (2007). The neutral detergent fibre with heat stable amylase and exclusive ash (aNDFom) contents were determined as per Van Soest *et al.* (1991). The non-fibrous carbohydrates (NFC) were calculated as follows: $\text{NFC} = 100 - \text{CP} - \text{NDF} - \text{EE}$ (NRC 2007).

Statistical analyses: Observations were analyzed using R 3.3.2 (R Development Core Team 2016). The fermentation characteristics of the silage were analyzed by 2-way factorial analysis of variance (ANOVA), where additive, fermentation period, and additive × fermentation period were the fixed effects. When the F-value indicated significance ($P < 0.05$), further testing was performed using pair-wise t-tests to compare the additive's mean value in each period. Fermentation characteristics and chemical compositions of the silage at 60 days of fermentation were analyzed by one-way analysis of variance (ANOVA), and the mean values of the additives were compared using pair-wise t-tests. Bonferroni correction was used to detect the differences between the means for each data analysis.

RESULTS AND DISCUSSION

Fermentation quality: The silage pH and lactic acid levels (Fig. 1), VFA concentration (Fig. 2), DM losses and Fleig point (Fig. 3) were affected by the silage additive, fermentation period and their interaction. The application of the silage additive reduced the pH value throughout the fermentation period compared with that of the no additive treatment. The lowest pH value was found in the MIX treatment, followed by the FJLB, COM and CON treatments after 60 days of ensiling ($P < 0.05$; Table 1). The highest lactic acid production was in the MIX treatment, followed by the FJLB, COM and CON treatments throughout the fermentation period. After 60 days of fermentation, the MIX and FJLB silages had higher levels of lactic acid than the CON and COM silages ($P < 0.05$; Table 1). Although acetic acid production fluctuated in all the treatments throughout the fermentation period, the MIX treatment contained higher

Table 1. Fermentative quality of the total mixed ration (TMR) silage containing agricultural by-products after 60 days of ensiling

Item	Treatment				P value
	CON	FJLB	COM	MIX	
pH	7.5±0.12 ^a	5.4±0.11 ^c	6.2±0.26 ^b	4.5±0.06 ^d	<.001
Lactic acid (g/kg DM)	4.9±1.14 ^b	23.2±1.15 ^a	7.8±2.11 ^b	25.2±2.96 ^a	<.001
VFA (g/kg DM)					
Acetic acid	6.2±1.49 ^b	4.3±0.40 ^b	4.2±0.42 ^b	11.9±0.73 ^a	<.001
Propionic acid	2.0±0.36 ^b	1.1±0.22 ^{bc}	3.5±0.25 ^a	0.5±0.14 ^c	<.001
Butyric acid	19.7±1.93 ^a	10.0±1.44 ^b	21.9±1.69 ^a	1.3±0.45 ^c	<.001
NH ₃ -N (g/kg TN)	58.4±5.74	61.7±4.25	73.3±8.22	58.1±2.02	0.217
DM losses (% DM)	2.7 ^{ab} ±0.61	1.7 ^b ±0.33	3.9±0.45 ^a	0.9±0.55 ^b	0.003
Fleig point	11.2±5.04 ^d	95.7±4.36 ^b	61.7±9.76 ^c	134.8±2.97 ^a	<.001
IVDMD (%)	58.2±1.18	59.7±1.57	60.2±1.01	61.4±0.56	0.267

CON, control (no additive); FJLB, fermented juice of epiphytic lactic acid bacteria additive; COM, commercial silage additive; MIX, FJLB+COM; VFA, volatile fatty acid; NH₃-N, ammonia-nitrogen; DM, dry matter; IVDMD, *in vitro* dry matter digestibility. Values are the means±standard errors (n=5). The values with different superscripts within the same row are significantly different at the 5% level.

acetic acid levels than the other treatments at 60 days of fermentation ($P<0.01$). The MIX treatment showed the lowest production of propionic acid and butyric acid among all the treatments on day 60 ($P<0.05$). The NH₃-N content was affected by the silage additive during the initial 2 days of fermentation, but there was no difference afterwards.

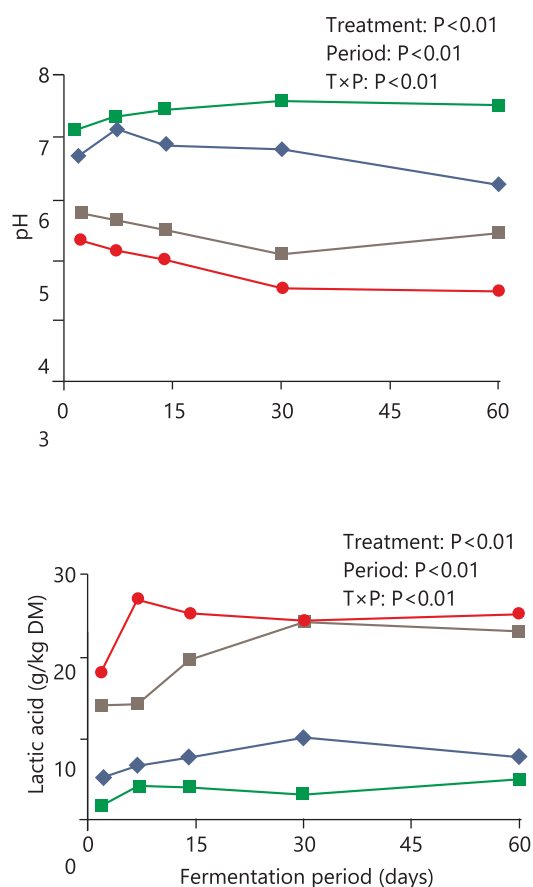


Fig. 1. The pH and lactic acid concentrations in the TMR silages prepared from agricultural and food by-products ensiled with the Control (■), FJLB (■), Commercial (◆), and Mix (●) additive treatments. The data were collected at 2, 7, 14, 30, and 60 days of fermentation. DM, Dry matter.

There was no interaction effect with the NH₃-N content throughout the 60 days of fermentation ($P>0.05$). The DM losses did not differ across all of the silage additive treatments before 30 days of fermentation. However, the MIX treatment showed lower DM losses than the CON treatment after 60 days of fermentation ($P<0.01$). The TMR with the FJLB treatment also showed lower DM losses at 60 days into the ensiling period compared to that with the COM treatment ($P<0.05$). The Fleig point in the MIX treatment was the highest of all the treatments until the last day of fermentation ($P<0.01$). The IVDMD was not affected by the silage additives, fermentation period or their interaction (Fig. 4).

The silage with the FJLB additive had a lower pH, and the highest lactic acid concentration was observed in the MIX treatment and might suggest that the lactic acid bacteria in the commercial product would result in better a fermentation quality when combined with the lactic acid bacteria in the FJLB but not when applied alone. In the present study, the FJLB treatment also had a lower pH than occurred with COM treatment, suggesting that the LAB in the FJLB was more suitable for TMR silage prepared from agricultural and food by-products. According to McDonald *et al.* (1991), pH value of well-preserved silages is ranged from 3.7 to 4.2. In the present study, the pH values in all treatments were higher than the recommendation value. It might be caused by low soluble carbohydrate content of agricultural by-products.

The FJLB treatment had much better lactic acid production than COM treatment, showing that LAB species of FJLB produced lactic acid more rapidly. The LAB is reported to produce lactic acid (McDonald *et al.* 1991) and that various species of lactic acid bacteria produce different levels of lactic acid from varying feed stocks (Burenook *et al.* 2005, Wang *et al.* 2009). Thus, the LAB species of FJLB produced lactic acid more rapidly, mainly after 7 days of fermentation, than that in the COM treatment, which is a commercial additive containing only 2 strains of lactic acid bacteria: *Lactococcus lactis* and *Lactobacillus paracasei*.

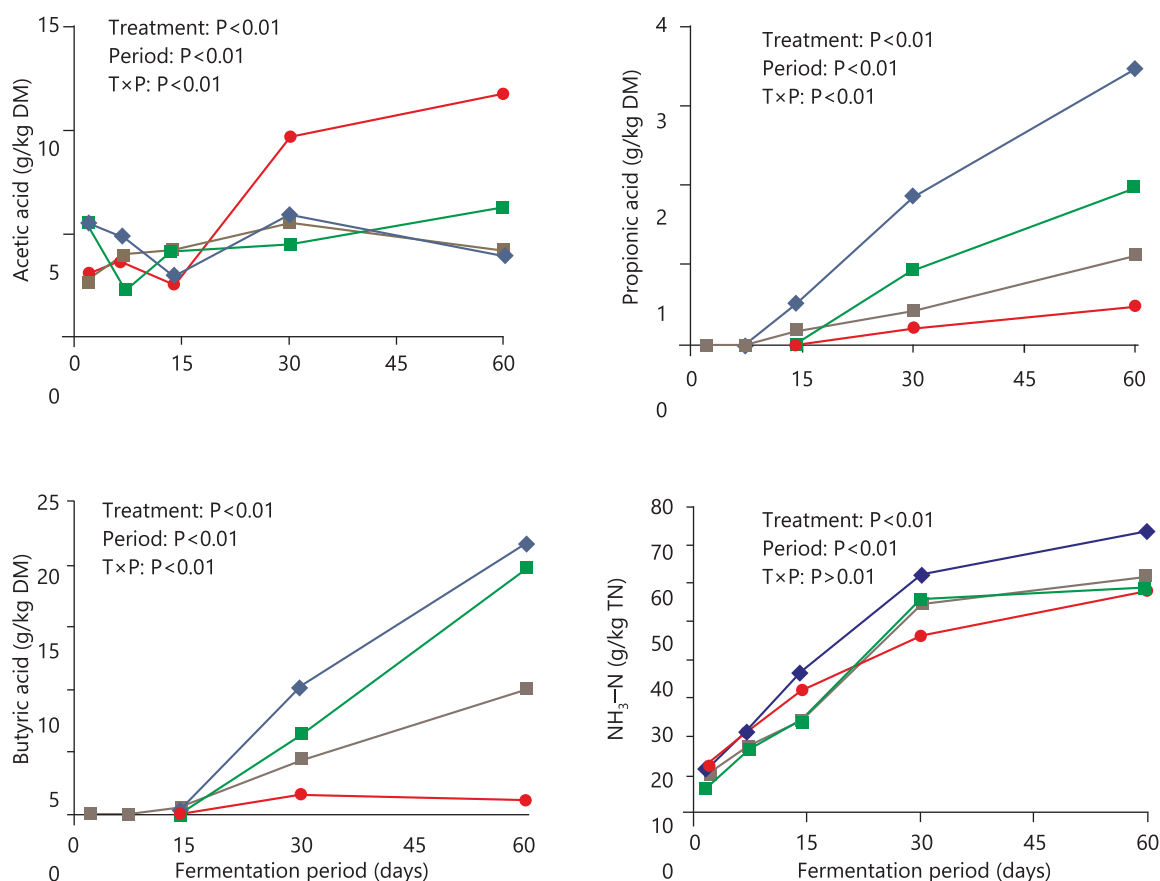


Fig. 2. The volatile fatty acid (VFA) and ammonia-nitrogen (NH₃-N) concentrations in the TMR silages prepared from agricultural and food by-products ensiled with the Control (■), FJLB (■), Commercial (◆), and Mix (●) additive treatments. The data were collected at 2, 7, 14, 30 and 60 days of fermentation. DM, dry matter; TN, total nitrogen.

These 2 strains of lactic acid bacteria in commercial additives might not be suitable in silage prepared from agricultural by-products. Present results are consistent with the previous reports of Tao *et al.* (2017) and Wang *et al.* (2009), who used FJLB as a silage additive to alfalfa, which yielded better fermentation quality than the inoculant of LAB additive. The recommendations for lactic acid content

in well-preserved silages ranged from 80 to 120 g/kg DM (McDonald *et al.* 1991). In the present study, all treatments showed lower lactic acid concentration than those suggested values. Again, it caused by lower soluble carbohydrates content in agricultural by-product.

Acetic acid is produced by heterofermentative bacteria, such as *Lactobacillus buchneri*, by converting lactic acid

Table 2. Chemical composition (g/100 g DM) of the total mixed ration (TMR) silage fermented with different types of silage additives after 60 days of ensiling

Chemical composition	Treatment				P value
	CON	FJLB	COM	MIX	
DM	53.5±0.34 ^{ab}	54.1±0.18 ^a	52.9±0.25 ^b	54.5±0.30 ^a	0.004
CP	13.9±0.19 ^b	14.1±0.31 ^{ab}	14.1±0.20 ^{ab}	15.0±0.16 ^a	0.016
aNDFom	53.1±1.01 ^a	53.6±0.65 ^a	52.5±0.49 ^a	47.7±0.77 ^b	<.001
ADFom	31.4±0.78 ^a	29.5±0.43 ^{ab}	30.9±0.52 ^{ab}	28.9±0.47 ^b	0.021
EE	2.8±0.04 ^c	3.3±0.09 ^{bc}	3.8±0.21 ^b	5.8±0.09 ^a	<.001
Crude ash	6.2±0.13	6.1±0.03	6.3±0.16	6.3±0.18	0.565
NFC	24.0±1.01	23.0±0.83	23.3±0.69	25.3±0.42	0.181

CON, control (no additive); FJLB, fermented juice of epiphytic lactic acid bacteria additive; COM, commercial silage additive; MIX, FJLB+COM; DM, dry matter; CP, crude protein; aNDFom, α-amylase neutral detergent fiber exclusive ash; ADFom, acid detergent fiber exclusive ash; EE, ether extract; NFC, non-fibrous carbohydrates (NFC=100CP-EE-aNDFom-crude ash). Values are the means±standard errors (n=5). The values with different superscripts within the same row are significantly different at the 5% level.

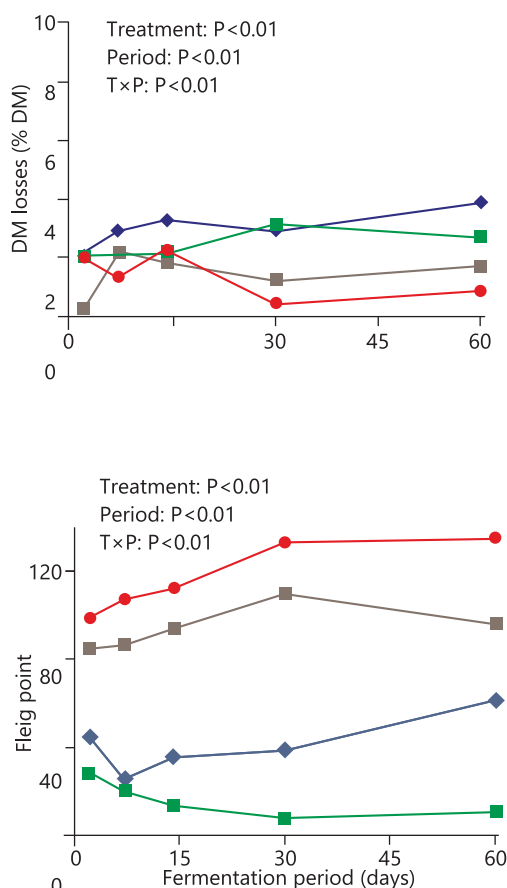


Fig. 3. The dry matter (DM) losses and Fleig point in the TMR silages prepared from agricultural and food by-products ensiled with the Control (■), FJLB (■), Commercial (◆), and Mix (●) additive treatments. The data were collected at 2, 7, 14, 30, and 60 days of fermentation.

to acetic acid under anaerobic conditions (Bureenok *et al.* 2016). Acetic acid is also responsible for the acidity of feed silage and is able to improve aerobic stability (Yitbarek and Tamir 2014) by decreasing the opportunistic bacteria and fungi (Wang *et al.* 2014). In the present study, the acetic acid production fluctuated across all of the treatments throughout the fermentation period. The highest acetic acid levels at 60 days were recorded in the MIX treatment, which also had the lowest pH value. Extending the duration of the fermentation may increase the acetic acid level (Minh *et al.* 2014).

The maximum level of butyric acid in silage that is accepted as good fermentation is 2 g/kg DM (Zhang *et al.* 2013). The concentration of the butyric acid in the FJLB treatment was lower than the maximum level observed over the first 14 days of fermentation and then increased rapidly afterwards. A possible reason in the FJLB treatment, the environment of the silage was not sufficiently acidic to restrict clostridia growth after 14 days of ensiling; the growth of clostridia is inhibited at a pH of 4.2 (McDonald *et al.* 1991). Usually, the butyric acid concentration in FJLB-treated silage is very low or not detectable, as reported in other studies (Horiguchi *et al.* 2008; Denek *et al.* 2011, 2012).

However, Wang *et al.* (2009) reported that the butyric acid concentration of the FJLB treatment reached more than 2 g/kg DM even on the first days of ensiling and then increased steadily during the fermentation period of alfalfa silage.

The MIX and FJLB treatments had a higher ammonia-nitrogen content than the control treatment on the second day of fermentation, but there was no significant difference afterwards among the treatments. A possible proteolytic microbes were present in the TMR silage during the first 2 days of fermentation since the pH (more than 4.5) was not sufficiently acidic to inhibit the growth of such undesirable microbes (Zhang and Yu 2017). However, the ammonia-nitrogen values in the present study were accepted since the values were less than 100 g/kg (Bureenok *et al.* 2016). This result is in line with the reports of FJLB on stylo legume and Guinea grass (Bureenok *et al.* 2016) and on alfalfa (Koc *et al.* 2016).

DM losses occurred as a result of microbial metabolism in the silage. The DM losses were not different across all of the silage additive treatments during the first 30 days of fermentation possibly microorganisms in all treatments degraded the nutrients in the TMR to a similar extent. After that, the environment of the silage in the MIX treatment, which was more acid than other treatments, successfully inhibited the unwanted microorganisms. These results are in agreement with Zhang *et al.* (2015), who reported reduction of silage pH due to reduction in the loss of simple carbohydrates by unwanted bacteria. This findings also explain the lower DM losses in the FJLB treatment in the present study.

The Fleig point is one tool used to evaluate silage quality (Ziaei and Molaei 2010). Fleig points from 85 to 100 indicate very good quality; 60 to 80, good quality; 55 to 60, moderate quality; 25 to 40, satisfactory quality, and <20, worthless quality (Denek and Can 2006). In the present study, the MIX treatment showed an excellent quality of fermentation followed by the FJLB treatment. The COM treatment was classified as moderate quality, and the TMR silage with no additive had a poor quality.

The IVDMD of the TMR silage was not affected by the addition of the silage additives, the fermentation period or their interaction. These findings are in contrast with reports of Nishino and Uchida (1999), who reported increased *in vitro* digestibility of the silage applied FJLB to lucerne. Wang *et al.* (2009) also reported higher IVDMD with the addition of FJLB to alfalfa silage. This suggested that applying FJLB to silage has various effects on the IVDMD as several factors affect the *in vitro* digestibility of silage, such as the concentration of fiber components. In this study, the TMR was prepared mainly from rice straw and corn cobs with a high fiber content. In rice straw, lignin and silica are the most important factors limiting digestibility (Van Soest 2006).

Chemical composition: The DM, CP, aNDFom, ADFom and EE contents were affected by the silage additive treatments (Table 2). The addition of the FJLB alone or in combination with the commercial LAB resulted in a higher

DM content than addition of the COM additive ($P < 0.04$). This result was consistent with other studies (Nishino and Uchida 1999, Denek *et al.* 2011), and suggested that the LAB in the FJLB were suitable for inhibiting the activity of undesirable microorganisms that were responsible for the nutrient losses. The FJLB and COM treatment had similar CP content to that of the untreated TMR silage. This result was consistent with a previous report that found that the FJLB additive did not improve the CP content in lucerne silage (Denek *et al.* 2011) or Napier grass (Bureenok *et al.* 2012). The lactic acid bacteria in the MIX treatment were more effective in restricting proteolysis during fermentation than that in the FJLB and COM treatments, because the pH decline rate in silages is important in determining the extent of proteolysis (McDonald *et al.* 1991). The aNDFom contents in the FJLB and COM treatments were not different from that in the CON treatment. However, the lowest aNDFom content was observed in the MIX treatment. This result suggested that the LAB from the FJLB and COM had a synergetic effect in reducing the fiber content of the TMR silage prepared from agricultural and industrial by-products. Reducing fiber content by using lactic acid bacteria or juice containing lactic acid bacteria have also been reported in some studies (Yahaya *et al.* 2004, Denek *et al.* 2011, 2012). However, the reason for this result is unclear and still needs to be investigated (Guo *et al.* 2014). Clear changes were observed in the EE content of the TMR silage. The EE content in the MIX treatment was the highest among the treatments. This result suggested that the low pH of the MIX treatment might have inhibited the degradation of the EE. In contrast, in the CON, FJLB and COM treatments, the acidity was not high enough to decrease the metabolism of crude fat by undesirable microorganisms including yeast and mold. Further investigation is needed to clarify this result.

In conclusion, low pH, high lactic acid, low butyric acid, low DM losses and higher Fleg points in the silage indicated that the application of the FJLB as a silage additive in the TMR silage prepared from the agricultural and industrial by-products improved the fermentation quality. Although the combination of the FJLB and the commercial additive treatments further improved the fermentation quality and nutritive values of the TMR silage compared with the FJLB treatment, such a combined approach would not be cost-effective when applied at scale to any farm. Further studies are needed to test the effects of FJLB as a silage additive in agricultural by-products on animal production.

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